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(19) (CA) **APPLICATION FOR CANADIAN PATENT** (12)

(54) Inhibition of Ketol-Acid Reductoisomerase by Oxalylhydroxamate Derivatives

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(57) 23 Claims

TITLE

INHIBITION OF KETOL-ACID REDUCTOISOMERASE BY OXALYLHYDROXAMATE DERIVATIVES

ABSTRACT

A process for the selective inhibition of ketol-acid reductoisomerase (EC 1.1.1.86; KARI) through the use of oxalylhydroxamate derivatives, thereby effecting herbicidal activity on plants and inhibiting microbial growth, is disclosed. Specific oxalylhydroxamate compounds are also disclosed. Additionally, a process for assaying ketol-acid reductoisomerase at low levels is part of the invention.

CR-8686

TITLE

INHIBITION OF KETOL-ACID REDUCTOISOMERASE BY OXALYLHYDROXAMATE DERIVATIVES

BACKGROUND OF THE INVENTION

Field of the Invention

This invention relates to the process of selectively inhibiting ketol-acid reductoisomerase (EC 1.1.1.86; KARI) through the use of oxalylhydroxamate derivatives, thereby effecting herbicidal activity on plants and inhibiting microbial growth. The invention also discloses specific oxalylhydroxamate compounds. Additionally, a process for assaying ketol-acid reductoisomerase at low levels is disclosed and can be used to assess the activity of inhibitors of this enzyme.

Summary of the Background

Plants, unlike animals, are able to synthesize the branched-chain amino acids. These amino acids are essential for animals because they lack the enzymes necessary for branched-chain amino acid synthesis and must therefore obtain such amino acids from dietary sources. Plants, however, contain three common enzymes specific to the synthesis of valine, leucine, and isoleucine, which are the branched-chain amino acids. The first of these sequential enzymes is acetolactate synthase, the second is ketol-acid reductoisomerase, and the third is dihydroxyacid dehydratase. Isoleucine biosynthesis requires a fourth enzyme, threonine deaminase, which provides a substrate for the first common enzyme, acetolactate synthase. Three additional enzymes are required for leucine biosynthesis, and these are isopropylmalate synthase, isopropylmalate dehydratase, and isopropylmalate dehydrogenase. The biosynthesis of all three branched-chain amino acids

also requires a transaminase, which is not restricted to branched-chain amino acid synthesis nor is it unique to plants.

It is known that selective inhibition of the first enzyme in the biosynthetic pathway for branched-chain amino acids, acetolactate synthase (EC 4.1.3.18), is the basis for the growth inhibition of plants, bacteria, yeast, and fungi by various sulfonylurea and imidazolinone herbicides. LaRossa, et al., J. Biol. Chem. 259:8753 (1984); Falco, et al., Genetics 109:21 (1985); Chaleff, et al., Science 224:1443 (1984); Ray, Plant Physiol. 75:827 (1984); Shaner, et al., Plant Physiol. 76:545 (1984); Muhitch et al., Plant Physiol. 83:451 (1987). Although the sulfonylurea and imidazolinone herbicides are quite selective in inhibiting acetolactate synthase, the site on this enzyme to which the herbicides bind is not intrinsically required for function. Schloss, et al., Nature (London) 331:360 (1988). This increases the likelihood that resistance to these herbicides in weeds will be obtained by the mutation of this enzyme.

Ketol-acid reductoisomerase (EC 1.1.1.86; KARI) is the second common enzyme specific to the biosynthesis of branched-chain amino acids for plants, bacteria, yeast, and fungi. There are currently no disclosed compounds known to be selective inhibitors of this enzyme. This invention discloses a process for the selective inhibition of ketol-acid reductoisomerase through the use of oxalylhydroxamate derivatives. Selective inhibition of ketol-acid reductoisomerase results in herbicidal activity and inhibition of microbial growth, and yet is not injurious to animals because animals lack this enzyme.

Ketol-acid reductoisomerase is magnesium dependent. Hydroxamates without the oxalyl moiety are known to

inhibit certain metal-dependent enzymes. Hydroxamate substrate and reaction-intermediate analogs are known to inhibit lipoxygenase (iron dependent), aminopeptidases (zinc dependent), collagenase (zinc dependent), elastase (zinc dependent), thermolysin (zinc dependent), urease (nickel dependent), and enolase (magnesium dependent). See, respectively, Summers, et al., J. Med. Chem. 30:574 (1987); Corey, et al., J. Am. Chem. Soc. 106:1503 (1984); Cherot, et al., Mol. Pharmacol. 30:338 (1986); Fournie, et al., J. Med. Chem. 28:1158 (1985); Chan, et al., J. Biol. Chem. 257:7955 (1982); Wilkes, et al., J. Biol. Chem. 258:13517 (1983); Baker, et al., Biochemistry 22:2098 (1983); Coletti-Previero, et al., Biochem. Biophys. Res. Commun. 107:465 (1982); Blumberg, et al., Life Sci. 28:301 (1981); Moore, et al., Biochem. Biophys. Res. Commun. 136:390 (1986); Kessler, et al., Infect. Immun. 38:716 (1982); Holmes et al., Biochemistry 22:236 (1983); Holmes, et al., Biochemistry 20:6912 (1981); Nishino, et al., Biochemistry 17:2846 (1978); Yamaya, et al., Plant Physiol (Bethesda) 67:1133 (1981); Nagarajan & Fishbein, Fed. Proc. 36:700 (1977); Kobashi, et al., Biochim. Biophys. Acta 227:429 (1971); and Anderson, et al., Biochemistry 23:2779 (1984).

The inhibition reactions of lipoxygenase, aminopeptidases, collagenase, elastase, and thermolysin are thought to proceed by reaction-intermediate mimicry. In the case of urease, the hydroxamate inhibitor acts as a nonspecific, metal chelator, and the particular structure of the hydroxamate is unimportant for this reaction, unlike the structural requirements for ketol-acid reductoisomase inhibition. Inhibition of enolase, which is a magnesium-dependent enzyme (as is ketol-acid reductoisomerase), probably depends on both metal chelation and reaction-intermediate mimicry. The structural requirements for the hydroxamate inhibitor of

enolase (phosphonoacetohydroxamate) differ from those of ketol-acid reductoisomerase, however, and the phosphonoaceto moiety is essential for inhibition of enolase. As discussed below, the inhibition of ketol-acid reductoisomerase requires the presence of an oxalyl moiety on the hydroxamate for potency.

A hydroxamate is also known to be an inhibitor of an enzyme that is not metal dependent, as is the case for the inhibition of triose phosphate isomerase by the hydroxamate of phosphoglycollate, wherein the hydroxamate presumably mimics the ene-diol reaction intermediate of this enzyme as the sole basis for inhibition. Collins, J. Biol. Chem. 249:136 (1974).

Specific instances of the inhibition of particular enzymes, other than ketol-acid reductoisomerase, by oxalyl- and oxamylhydroxamates are are known in the art. Oxamylhydroxamate (H_2N-CO-CO-NH-OH) inhibits virus, bacterial, and tumor cell replication at high ($> 10^{-4}$ M) concentrations. Gale et al., Experientia (Basel) 24:194 (1968); Hynes, et al., J. Med. Chem. 16:576 (1973). The mode of action is thought to be similar to hydroxyurea, which acts as a radical scavenger, inactivating ribonucleotide reductase, a radical-containing enzyme essential to the biosynthesis of deoxynucleotides (DNA biosynthesis). Gale, Cancer Res. 26:2340 (1966); Kjoeller Larsen, et al., Eur. J. Biochem. 125:75 (1982). Thus, the reaction does not proceed by metal chelation or reaction-intermediate mimicry. (That oxamylhydroxamate inhibits growth by virtue of its inhibition of ribonucleotide reductase is consistent with the observation that deoxyribonucleosides protect Ehrlich ascites tumor cells against inhibition. Gale, Experientia (Basel) 24:57 (1968).) This radical-scavenger mode of action is probably shared by several derivatives of oxamylhydroxamate, in which the amide

nitrogen bears various alkyl or aryl substituents (R-NH-CO-CO-NH-OH), that have been described as having fungicidal (R = 4-tolyl) or bactericidal (R = $PhCH_2$-) activity. Petyunin, et al., Khim. Farm. Zh. 12:106 (1978).

Oxamylhydroxamate is also known to inhibit histidine decarboxylase and glutamate carboxylyase. Gale, et al., Biochem. Pharmacol. 19:628 (1970); Howle & Gale, Proc. Soc. Exp. Biol. Med. 131:697 (1969). Inhibition of histidine decarboxylase and glutamate carboxylyase is not dependent on the oxalyl moeity of oxamylhydroxamate, because a number of other structurally unrelated hydroxamates are equally effective as inhibitors. Similarly, N-methyl oxalylhydroxamate methyl ester, which is one of the inhibitors of ketol-acid reductoisomerase as disclosed in the present invention, has been described as an inhibitor of urease, but it is no better an inhibitor than are a variety of other hydroxamates. Kobashi, et al., Biochim. Biophys. Acta 227:429 (1971). This indicates that the oxalyl moiety is not critical to urease inhibition, which differentiates it from the selective ketol-acid reductoisomerase inhibition, wherein the oxalyl moiety is an essential feature for potent inhibition. As discussed above, the inhibition reaction for urease is thought to proceed through nonspecific metal chelation, whereas the suggestion in the present invention is that oxalylhydroxamate derivatives inhibit ketol-acid reductoisomerase by, in part, their metal chelation properties and, to a greater extent, by their mimicry of the reaction intermediate of the rearrangement catalyzed by ketol-acid reductoisomerase.

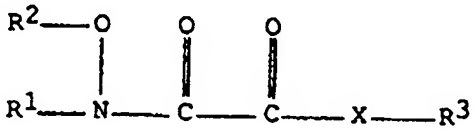
None of the other oxalylhydroxamates has been previously described as an enzyme inhibitor or as an

inhibitor of a biological process. Additionally, the present invention discloses specific oxalylhydroxamate derivatives that currently are not known in the art. Oxalylhydroxamate, oxalylhydroxamate methyl ester, and N-methyl oxalylhydroxamate methyl ester have been previously disclosed. Kobashi, et al., Biochim. Biophys. Acta 227:429 (1971).

Ketol-acid reductoisomerase can be assayed from purified bacterial preparations, as described by Arfin, et al., J. Biol. Chem. 244:1118 (1969). The present invention discloses a process for assaying ketol-acid reductoisomerase at low levels through the use of selectively radiolabeled substrates.

SUMMARY OF THE INVENTION

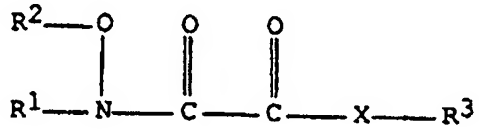
The present invention provides a process for inhibiting the enzymatic activity of ketol-acid reductoisomerase to effect herbicidal activity or to inhibit microbial growth, which comprises contacting, respectively, a plant or microorganism with an effective amount of a chemical compound of the formula



or an agriculturally acceptable salt thereof, wherein R^1 is selected from the group consisting of H, alkyl, alkenyl, alkynyl, aryl, alkylaryl, alkenylaryl, and alkynylaryl substituents having from 1 to about 300 atoms, wherein one or more carbon atoms can be optionally substituted with a heteroatom selected from the group consisting of N, S, O, Si, and P, and wherein one or more hydrogen atoms can be optionally substituted with a substituent selected from the group consisting of F, Cl, Br, I, and OH; R^2 is selected from the group

consisting of the group consisting of H, alkyl, alkenyl, alkynyl, aryl, alkylaryl, alkenylaryl, and alkynylaryl substitutents having from 1 to about 50 atoms, and acyl substituents having from 1 to about 300 atoms, wherein one or more carbon atoms can be optionally substituted with a heteroatom selected from the group consisting of N, S, O, Si, and P, and wherein one or more hydrogen atoms can be optionally substituted with a substituent selected from the group consisting of F, Cl, Br, I, and OH; R^3 is selected from the group consisting of H, alkyl, alkenyl, alkynyl, aryl, acyl, alkylaryl, alkenylaryl, and alkynylaryl substitutents having from 1 to about 300 atoms, wherein one or more carbon atoms can be optionally substituted with a heteroatom selected from the group consisting of N, S, O, Si, and P, and wherein one or more hydrogen atoms can be optionally substituted with a substituent selected from the group consisting of F, Cl, Br, I, and OH; and X is selected from the group consisting of O, S, and NY, wherein Y is selected from the group consisting of H, OH, and independently selected R^3; provided that the combination of X and R^3 is hydrolytically labile.

Additionally, the present invention discloses chemical compounds of the formula



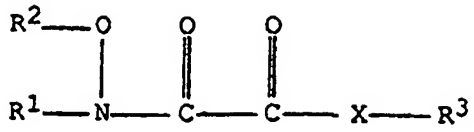
wherein R^1 is selected from the group consisting of H, methyl, ethyl, propyl, isopropyl, butyl, sec-butyl, isobutyl, t-butyl, phenyl, 2-pentyl, 3-pentyl, 2-hexyl, 3-hexyl, C1-C6 perfluoroalkyls, and benzyl wherein one or more o-, m-, or p- positions can be optionally substituted with a substituent selected from the group

consisting of F, Cl, Br, I, methoxy, methyl, and trifluoromethyl; R^2 is selected from the group consisting of H, methyl, ethyl, propyl, isopropyl, butyl, sec-butyl, isobutyl, t-butyl, carbamoyl, N,N-dimethylcarbamoyl, N,N-dimethylthiocarbamoyl, N,N-diphenylcarbamoyl, N,N-diphenylthiocarbamoyl, acetyl, thioacetyl, propanoyl, thiopropanoyl, trimethylsilyl, t-butyldimethylsilyl, and benzoyl wherein one or more o-, m-, or p- positions can be optionally substituted with a substituent selected from the group consisting of F, Cl, Br, I, methoxy, methyl, and trifluoromethyl; R^3 is selected from the group consisting of H, methyl, propyl, isopropyl, butyl, sec-butyl, isobutyl, t-butyl, 2-pentyl, 3-pentyl, 2-hexyl, 3-hexyl, and C1-C6 perfluoroalkyls; and X is selected from the group consisting of O and S; provided that when R^1 is H, R^2 cannot be H; and further provided that when R^2 is H, R^1 and R^3 cannot both be CH_3.

The present invention also includes a process for assaying ketol-acid reductoisomerase, which comprises mixing an aliquot selected from the group consisting of a plant extract and a microbial extract with radioactive [1-^{14}C]-acetolactate, NADPH, and Mg^{2+}, in a buffered solution having a pH above about 6 and below about 9; adding an acid to the assay mixture, after an appropriate incubation time; removing volatile components from the acidified mixture; and measuring the remaining radioactivity.

DETAILED DESCRIPTION OF THE INVENTION

The present invention provides processes for inhibiting the enzymatic activity of ketol-acid reductoisomerase to effect herbicidal activity or to inhibit microbial growth, which comprises contacting, respectively, a plant or microorganism with an effective amount of a chemical compound of the formula



or an agriculturally acceptable salt thereof, wherein R^1 is selected from the group consisting of H, alkyl, alkenyl, alkynyl, aryl, alkylaryl, alkenylaryl, and alkynylaryl substituents having from 1 to about 300 atoms, wherein one or more carbon atoms can be optionally substituted with a heteroatom selected from the group consisting of N, S, O, Si, and P, and wherein one or more hydrogen atoms can be optionally substituted with a substituent selected from the group consisting of F, Cl, Br, I, and OH; R^2 is selected from the group consisting of the group consisting of H, alkyl, alkenyl, alkynyl, aryl, alkylaryl, alkenylaryl, and alkynylaryl substitutents having from 1 to about 50 atoms, and acyl substituents having from 1 to about 300 atoms, wherein one or more carbon atoms can be optionally substituted with a heteroatom selected from the group consisting of N, S, O, Si, and P, and wherein one or more hydrogen atoms can be optionally substituted with a substituent selected from the group consisting of F, Cl, Br, I, and OH; R^3 is selected from the group consisting of H, alkyl, alkenyl, alkynyl, aryl, acyl, alkylaryl, alkenylaryl, and alkynylaryl substitutents having from 1 to about 300 atoms, wherein one or more carbon atoms can be optionally substituted with a heteroatom selected from the group consisting of N, S, O, Si, and P, and wherein one or more hydrogen atoms can be optionally substituted with a substituent selected from the group consisting of F, Cl, Br, I, and OH; and X is selected from the group consisting of O, S, and NY, wherein Y is selected from the group consisting of H, OH, and

independently selected R^3; provided that the combination of X and R^3 is hydrolytically labile.

The combination of X and R^3 must be hydrolytically labile, wherein X-R^3 will be substituted by OH in the presence of water, either spontaneously or through the action of naturally occurring esterases and amidases. When R^2 is an acyl substituent, the number of atoms is not critical to the process of the present invention because the acyl substituent is hydrolytically labile and ultimately will be cleaved from the oxalylhydroxamate and will be replaced by H. Because R^1 and R^2 are near to each other, there is competition for space and, the larger the R^1 substituent, the more R^2 will be constrained in size, and vice versa. The alkyl, alkenyl, alkynyl, acyl, alkylaryl, alkenylaryl, and alkynylaryl substituents of R^1, R^2, and R^3 may be linear, branched, cyclic, or mixtures thereof. The alkenyl and alkenylaryl substituents have one or more unsaturated positions (double bonds). The alkynyl and alkynylaryl substituents have one or more triple bonds.

Optionally, R^1 can be joined to either R^2 or R^3 to form a ring; R^2 can be joined to either R^1 or R^3 to form a ring; or R^1 and R^2 and R^3 can be joined to form two fused rings.

A preferred embodiment is wherein R^1 has from 1 to about 150 atoms, with 1 to about 100 atoms being more preferred, and with 1 to about 50 atoms being most preferred; R^2 has from 1 to about 50 atoms, with 1 to about 30 atoms being more preferred, and with 1 to 20 atoms being most preferred; and R^3 has from 1 to about 150 atoms, with 1 to about 100 atoms being more preferred, and with 1 to about 50 atoms being most preferred.

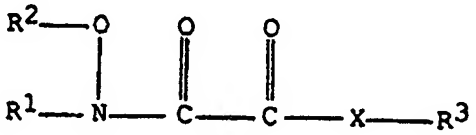
Preferably, X is O. Preferred oxalylhydroxamate derivatives for the process of the present invention

include compounds wherein R^1 is selected from the group consisting of hydrogen, methyl, ethyl, isopropyl, t-butyl, and benzyl, with hydrogen, methyl, ethyl, isopropyl, and benzyl being most preferred. Another preferred embodiment is wherein R^2 is selected from the group consisting of hydrogen, methyl, and t-butyl, with hydrogen being most preferred. Preferred R^3 substituents are those selected from the group consisting of hydrogen and methyl, with hydrogen being most preferred.

The most preferred compounds for the process of this invention are, in descending order of preference, N-isopropyl oxalylhydroxamate (wherein R^1 is isopropyl, R^2 and R^3 are H, and X is O), N-ethyl oxalylhydroxamate (wherein R^1 is ethyl, R^2 and R^3 are H, and X is O), and N-methyl oxalylhydroxamate (wherein R^1 is methyl, R^2 and R^3 are H, and X is O).

An effective amount of the chemical compounds discussed above is contacted with a plant or a microorganism to result in, respectively, herbicidal activity or inhibition of microbial growth. By an effective amount, it is meant that amount causing 50 % or greater mortality in plants or resulting in readily observable toxic effects, such as chlorosis, leaf curling, and leaf burning, or, respectively, that amount causing a greater than three-fold increase in the time required for microbial cell reproduction.

Additionally, the present invention discloses chemical compounds of the formula



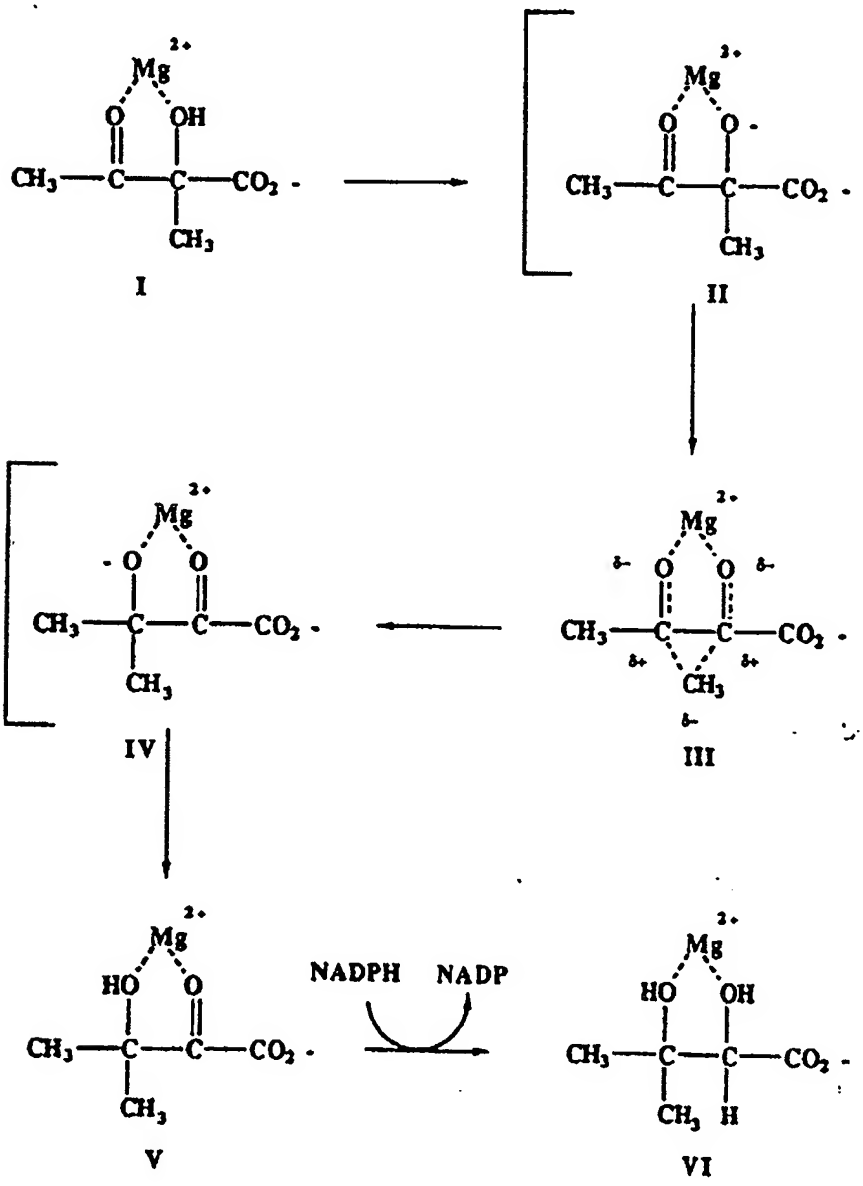
wherein R^1 is selected from the group consisting of H, methyl, ethyl, propyl, isopropyl, butyl, sec-butyl, isobutyl, t-butyl, phenyl, 2-pentyl, 3-pentyl, 2-hexyl, 3-hexyl, C1-C6 perfluoroalkyls, and benzyl wherein one or more o-, m-, or p- positions can be optionally substituted with a substituent selected from the group consisting of F, Cl, Br, I, methoxy, methyl, and trifluoromethyl; R^2 is selected from the group consisting of H, methyl, ethyl, propyl, isopropyl, butyl, sec-butyl, isobutyl, t-butyl, carbamoyl, N,N-dimethylcarbamoyl, N,N-dimethylthiocarbamoyl, N,N-diphenylcarbamoyl, N,N-diphenylthiocarbamoyl, acetyl, thioacetyl, propanoyl, thiopropanoyl, trimethylsilyl, t-butyldimethylsilyl, and benzoyl wherein one or more o-, m-, or p- positions can be optionally substituted with a substituent selected from the group consisting of F, Cl, Br, I, methoxy, methyl, and trifluoromethyl; R^3 is selected from the group consisting of H, methyl, propyl, isopropyl, butyl, sec-butyl, isobutyl, t-butyl, 2-pentyl, 3-pentyl, 2-hexyl, 3-hexyl, and C1-C6 perfluoroalkyls; and X is selected from the group consisting of O and S; provided that when R^1 is H, R^2 cannot be H; and further provided that when R^2 is H, R^1 and R^3 cannot both be methyl.

An additional compound is disclosed wherein R^1 is ethyl and R^2 is carbamoyl and C-2 of ethyl and N of carbamoyl are joined to form a ring.

Preferred oxalylhydroxamate derivatives include compounds wherein R^1 is selected from the group consisting of isopropyl and ethyl, with ethyl being most preferred. Another preferred embodiment is wherein R^2 is H.

Synthesis of oxalylhydroxamate derivatives is given in Examples 2-3.

Turning to the inhibition mechanism of ketol-acid reductoisomerase, a possible reaction pathway for the enzyme is illustrated below.

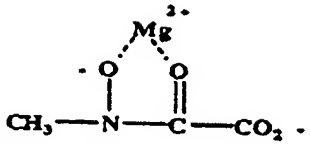


I II III IV V VI

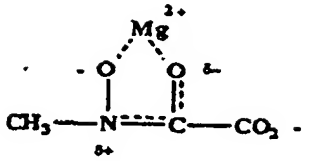
In this possible reaction pathway, one of the physiological substrates, α-acetolactate (I), is converted to the product 2,3-dihydroxyisovalerate (VI).

The other physiological substrate and product are α-aceto-α-hydroxybutyrate and 3-methyl-2,3-dihydroxypentanoate. The remaining structures (II-V) are tightly bound intermediates of the reaction, which never leave the enzyme. Although it is known that 2-oxo-3-methyl-3-hydroxybutanoate (V) is reduced by the enzyme when the independently synthesized compound is exogenously supplied to the enzyme, a concentration of this intermediate never builds up during the normal reaction, suggesting that it is tightly bound by the enzyme. One analog of intermediate (V), N-methyloxalylhydroxamate (VIIa, below), is of particular interest because it has characteristics (apparent in resonance form VIIb, below) similar to earlier intermediates in the reaction pathway (IV and III). Furthermore, as ketol-acid reductoisomerase is a magnesium-dependent enzyme, and magnesium is likely to be involved in the rearrangement of (I) into (V), the analog (VIIa, below) has the added advantage of being an excellent metal chelator. Thus, there is the potential for ketol-acid reductoisomerase inhibition by oxalyhydroxamates due to both reaction-intermediate mimicry and hydroxamate coordination of the essential magnesium.

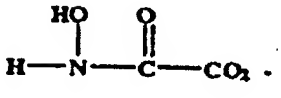
The important structural features for inhibition of ketol-acid reductoisomerase are best illustrated by considering the structures below.



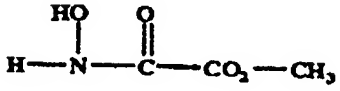
VIIa



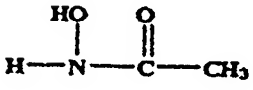
VIIb



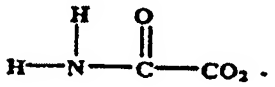
VIII



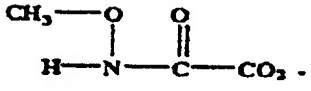
IX



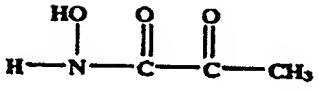
X



XI



XII



XIII

Substitutions on the nitrogen (i.e., R^1) of oxalylhydroxamate are well tolerated, as numerous substituents either increase the affinity of the enzyme for the compound relative to oxalylhydroxamate or reduce it only slightly (Examples 4-16). By contrast, substituents on the oxygens of the hydroxamate (XII) or carboxylate (IX) consistently reduce the enzyme's affinity for the compound (Example 4). However, elimination of either carboxylate or N-hydroxyl oxygen has a much more dramatic effect (i.e., 10^4- to 10^5-fold reduced affinity for the enzyme). Oxamate (XI) or pyruvyl hydroxamate (XIII) are not time-dependent

inhibitors of ketol-acid reductoisomerase, and the affinity of the enzyme for these analogs is reduced relative to their hydroxyl-bearing counterpart (VIII), by greater than 10^5 and 10^4, respectively (Example 4). Similarly, completely replacing the carboxylate of oxalylhydroxamate with a methyl moeity results in the rather poor inhibitor acetohydroxamate (X), which has an affinity reduced by greater than 10^5 relative to oxalylhydroxamate (Example 4). Structurally, an oxalyl moiety is necessary for ketol-acid reductoisomerase inhibition. Simple metal chelators or hydroxamates, such as compounds (X) and (XIII), will not potently inhibit this enzyme.

Herbicidal activity results when plants are brought in contact with the inhibitors of this invention at concentrations of between 10 and 100 ppm (Examples 23-30). The herbicidal oxalylhydroxamate compounds may be contained in the medium in which the plants are grown or may be applied to the surfaces of growing plants. Herbicidal action results in the lack of growth after germination. Topical applications at a rate greater than 0.4 kilograms/hectare may result in other symptoms of plant toxicity such as chlorosis, leaf burn, and curling of the leaves (Examples 38-46).

The process of the present invention inhibits microbial growth through the inhibition of ketol-acid reductoisomerase by oxalylhydroxamate derivatives. Bacterial growth is affected by the addition of filter-sterilized solutions of these oxalylhydroxamate derivatives to sterile growth media solutions lacking branched-chain amino acids. These solutions contain 0.02 % thiamine (required for Escherichia coli K strains) and 0.2 % glucose as a carbon source. An inoculum of bacteria, with sufficiently few bacteria in it so that the inoculated solution is initially almost

free of turbidity, is added. The turbidity of samples lacking inhibitor increases with time as a result of bacterial growth, which can be correlated with increases in bacterial numbers. Solutions containing greater than 1 mM of the oxalylhydroxamate inhibitors display no turbidity after 8 to 27 hours, and thus little or no bacterial growth has taken place. Lower concentrations of inhibitor allow some bacterial growth, albeit a time-dependent lag in cell growth is observed (Examples 17-20). Isoleucine and valine (branched-chain amino acids) may be added to eliminate the time-dependent lag in cell growth and, this demonstrates the selective inhibition of branched-chain amino acid biosynthesis in the intact organism (Examples 21-22).

The present invention also includes a process for assaying ketol-acid reductoisomerase, which comprises, in sum, mixing an aliquot selected from the group consisting of a plant extract and a microbial extract with radioactive [1-^{14}C]-acetolactate, NADPH, and Mg^{2+}, in a buffered solution having a pH above about 6 and below about 9; adding an acid to the assay mixture, after an appropriate incubation time; removing volatile components from the acidified mixture; and measuring the remaining radioactivity.

The ketol-acid reductoisomerase enzyme currently may be assayed from purified bacterial preparations, as described by Arfin et al., J. Biol. Chem. 244:1118 (1969), but this method is not well-suited to plant extracts because of the high level of enzyme required and the nonspecific oxidation of NADPH. The assay of the present invention is suitable with low levels of the enzyme and avoids the consequences of nonspecific oxidation of NADPH.

The present assay measures the time-dependent increase in acid-stable radioactivity due to the

reduction of [1-^{14}C]-acetolactate (acid-sensitive substrate, 48 cpm/nmol) to [1-^{14}C]-2,3-dihydroxyisovalerate (acid-stable product) by reductoisomerase in the presence of NADPH and magnesium (with liberation of $^{14}CO_2$).

The reaction is initiated by mixing an aliquot of a ketol-acid reductoisomerase-containing solution (containing 4 X 10^{-5} mmol/min of activity in 10 to 20 µL; more or less activity, in any convenient volume, can be used in the assay by changing the assay time proportionately) to a 1 mL assay solution (final volume) containing 100 mM HEPES-NaOH, pH 7.4, 10 mM $MgCl_2$, 400 µM NADPH, 125 µM [1-^{14}C]-acetolactate (48 cpm/nmole), and various concentrations of the oxalylhydroxamate inhibitor to be tested.

[1-^{14}C]-Acetolactate can be obtained by the conversion of [1-^{14}C]-pyruvate (Du Pont-New England Nuclear) by use of acetolactate synthase, as described by Schloss, et al., Biochemistry 24:4952 (1985). Alternatively, [1-^{14}C]-pyruvate can be obtained from D-ribulose 1,5-bisphosphate and $^{14}CO_2$ by the action of ribulosebisphosphate carboxylase, phosphoglycerate mutase, enolase, pyruvate kinase, ADP, Mg^{2+}, and 2,3-bisphosphoglycerate at pH 8. With the exception of $^{14}CO_2$ (Du Pont-New England Nuclear), these reagents can be obtained commercially from Sigma Chemical Co.

After incubating the samples for an appropriate time, which is the time necessary to give detectable levels of product and depends upon the specific activity and quantity of the radioactive material (herein 5 hours at 26°C), the enzymatic reaction is terminated and [^{14}C]-acetolactate is converted to acetoin and $^{14}CO_2$ by adding 0.1 mL 50% trifluoracetic acid.

The volatile components of the acidified solution are removed, preferably by evaporation at 100°C under a

stream of nitrogen. Other methods of removal include, e.g., drying, gassing, and prolonged standing. The resulting residue is resuspended in 50 % trifluoroacetic acid, and the suspension is re-evaporated. The final residue is resuspended in 1 mL water and 10 mL Scintiverse I® (Fischer Scientific Co., Pittsburgh, PA) is added, and the radioactivity is measured by scintillation counting.

The enzymatic activity of ketol-acid reductoisomerase is 50 % inhibited under these conditions with the preferred oxalylhydroxamate inhibitors (Examples 5-16) at concentrations between 9 and 20 nM for bacterial ketol-acid isomerase and between 50 and 100 nM for plant ketol-acid isomerase. The lowest concentration that yields 50 % inhibition of the enzyme is 9 nM and is approximately half of the enzyme concentration employed in the assay. This represents the theoretical limit for an exceptionally potent inhibitor, i.e., one molecule of inhibitor for each molecule of enzyme.

EXAMPLES

The following Examples illustrate, but do not limit, the processes and compounds of the present invention. Examples 1-3 illustrate the synthesis of various oxalylhydroxamates, with the claimed compounds in Examples 2-3. Example 4 assesses the time-dependent nature of the inhibition of ketol-acid reductoisomerase and also shows that inhibition of ketol-acid reductoisomerase by oxalylhydroxamate derivatives is functionally irreversible. Examples 5-16 disclose the process of the present invention for assaying ketol-acid reductoisomerase and demonstrate that both bacterial and plant enzymes are inhibited by oxalylhydroxamate derivatives. Examples 17-20 show that growth of bacteria is inhibited by oxalylhydroxamate derivatives.

Examples 21-22 demonstrate that inhibition of bacterial growth (a consequence of ketol-acid reductoisomerase inhibition) is prevented in the presence of branched-chain amino acids, and Examples 23-30 illustrate the herbicidal effect of oxalylhdroxamate derivatives on plants (a consequence of ketol-acid reductoisomerase inhibition) as well as the prevention of their herbicidal effect on plants by the presence of branched-chain amino acids. Examples 31-37 prove that the process of the present invention inhibits only ketol-acid reductoisomerase, and not one or more of the other common ezymes in the branched-chain amino acid biosynthetic sequence. Examples 38-46 demonstrate the post-emergence herbicidal activity in whole plants brought about by the inhibition of ketol-acid reductoisomerase using topical applications of oxalylhydroxamate derivatives.

The following abbreviations are employed throughout the Examples:

OHA: oxalylhydroxamate

KARI: ketol-acid reductoisomerase

NADP: nicotinamide adenine dinucleotide phosphate

NADPH: nicotinamide adenine dinucleotide phosphate, reduced form

FAD: flavin adenine dinucleotide

HEPES: N-2-hydroxyethyl piperazine-N'-ethane sulfonic acid

TRICINE: N-tris-(hydroxymethyl) methyl glycine

TLC: thin layer chromatography

Specific substituent values for compounds used in the Examples are:

oxalylhydroxamate (R^1 = H; R^2 = H; R^3 = H; X = O)

oxalylhydroxamate methyl ester (R^1 = H; R^2 = H; R^3 = CH_3; X = O)

N-methoxy OHA (R^1= H; R^2 = CH_3; R^3 = H; X = O)

N-methoxy OHA methyl ester (R^1 = H; R^2 = CH_3; R^3 = CH_3; X = O)

N-methyl OHA (R^1 = CH_3; R^2 and R^3 = H; X = O)

N-methyl OHA methyl ester (R^1 = CH_3; R^2 = H; R^3 = CH_3; X = O)

N-ethyl OHA (R^1 = C_2H_5; R^2 and R^3 = H; X = O)

N-ethyl OHA methyl ester (R^1 = C_2H_5; R^2 = H; R^3 = CH_3; X = O)

N-isopropyl OHA (R^1 = C_3H_7; R^2 and R^3 = H; X = O)

N-isopropyl OHA methyl ester (R^1 = C_3H_7; R^2 = H; R^3 = CH_3; X = O)

N-t-butyl OHA (R^1 = C_4H_9; R^2 and R^3 = H; X = O)

N-t-butyl OHA methyl ester (R^1 = C_4H_9; R^2 = H; R^3 = CH_3; X = O)

N-benzyl OHA (R^1 = C_7H_7; R^2 and R^3 = H; X = O)

N-benzyl OHA methyl ester (R^1 = C_7H_7; R^2 = H; R^3 = CH_3; X = O)

N-t-butoxy OHA (R^1 = H; R^2 = C_4H_9; R^3 = H; X = O)

N-t-butoxy OHA methyl ester (R^1 = H; R^2 = C_4H_9; R^3 = CH_3; X= O)

Example 1

Synthesis of Oxalylhydroxamate and Oxalylhydroxamate Methyl Ester

To 1 g of hydroxylamine hydrochloride (14.3 mmol) in 100 mL of methanol were added 4.01 mL of triethylamine (28.7 mmol) and 1.32 mL of methyloxalylchloride (14.3 mmol). After allowing the reaction to proceed for 30 minutes at room temperature, 1.43 mL of 10 N NaOH (14.3 mequivalents) were added. Three hours after the addition of base, the sample was diluted with an equal volume of water (pH = 9.9) and 1 mL of concentrated hydrochloric acid was added to adjust the pH to 6.5. Following dilution of the sample to 1 liter with water, it was applied to a 2.5 X 59 cm

column of AG1-X8 (Chloride form, BioRad Labs, Richmond, CA 94804). The column was eluted with 200 mL of water, followed by 2 liters of 50 mM HCl, and 23 mL fractions of the acid eluant were collected. Hydroxamates were detected by the addition of 0.2 mL of each fraction to 0.1 mL of 1 % $FeCl_3$ in 1 N HCl. Hydroxamates give a red color in this test and two hydroxamate peaks were detected, one contained in fractions 10-14, and another contained in fractions 41-55. The fractions containing each hydroxamate were pooled separately and lyophilized to give 159 mg of OHA methyl ester (first peak) and 103 mg of OHA (second peak). Titration of the OHA with 0.1 N KOH (to pH 7) gave an equivalent weight of 111 vs. the expected value of 105, with a pK of 2.4.

Example 2

Synthesis of N-Ethyl Oxalylhydroxamate and N-Ethyl Oxalylhydroxamate Methyl Ester

N-Ethylhydroxylamine was prepared from the $NaBH_3CN$ reduction of acetaldehyde oxime according to the procedure of Borch, et al., J. Am. Chem. Soc. 93:2897 (1971).

Methyl N-ethyl oxalylhydroxamate was prepared by the dropwise addition of a solution of 0.82 g N-ethylhydroxylamine, 1.87 ml triethylamine, and 20 ml chloroform to a solution of 1.64 g methyloxalylchloride dissolved in 100 mL diethyl ether at room temperature. The solution was stirred 2 hours then filtered to remove precipitated triethylamine hydrochloride salt. Evaporation under reduced pressure of solvent and unreacted triethylamine and methyloxalylchloride afforded 1.91 g of a viscous pale yellow oil. One spot with a R_f = 0.83 was observed on silica gel TLC plates using ethanol as a developing solvent, and it tested positive for hydroxamic acid with acidic $FeCl_3$. The ^1H

NMR in $CDCl_3$ corresponded to N-ethyl OHA methyl ester: δ 1.40 ppm (triplet, 3H), δ 3.90 (s, 3H), δ 3.96 (q, 2H).

N-Ethyl OHA was prepared by alkaline hydrolysis of 0.205 g N-ethyl OHA methyl ester in 10 ml water with 1 equivalent of potassium hydroxide. Hydrolysis was monitored by the decrease of the resonance at 3.90 ppm in the ^1H NMR spectrum.

N-Ethyl OHA and its methyl ester are compounds disclosed in the present invention.

Example 3

Synthesis of N-Methyl, N-Isopropyl, N-Methoxy, N-Benzyl, N-t-Butyl and N-t-Butoxy Oxalylhydroxamates and Their Methyl Esters

The methyl ester of N-benzyl OHA was prepared by the procedure described in Example 2 starting with N-benzaldehyde oxime. The methyl ester was a cream-colored solid (m.p. 102-104°C).

The methyl esters of N-methyl, N-isopropyl, N-methoxy, N-butyl and N-t-butoxy OHA were prepared by the procedure described in Example 2, with the exception that the hydrochloride salts of the hydroxylamines were initially dissolved in chloroform with two equivalents of triethylamine. This chloroform solution was added to methyloxalylchloride in diethyl ether. After 6 hours, the mixture was filtered to remove precipitated triethylamine hydrochloride salt, and the solvent was removed under reduced pressure. The methyl esters were purified by vacuum distillation.

compound	pressure (mm)	b.p. (°C)
N-methyl OHA	0.12	92-94
N-isopropyl OHA	0.10	95-97
N-t-butoxy OHA	0.09	74-76
N-t-butyl OHA	0.10	74-76

The corresponding acids were obtained as their potassium salts by alkaline hydrolysis of the methyl ester with one equivalent of potassium hydroxide.

All of the compounds in Example 3, with the exception of N-methyl OHA methyl ester, are disclosed as part of the present invention.

Example 4

Assessment of the Time-Dependent Nature of the Inhibition of Ketol-Acid Reductoisomerase by Use of a Continuous Assay

Inhibition of the purified *E. coli* KARI by all oxalyhydroxamate derivatives is time dependent. At a concentration of 0.1 μM of N-isopropyl OHA, 66 nM KARI was inactivated with a half-time of about 2 minutes when it was added last to an assay solution containing 100 mM HEPES, pH 7.4, 0.13 mM acetolactate, 10 mM $MgCl_2$, and 0.2 mM NADPH. The assay was continuously monitored at 340 nm by use of a recording spectrophotometer. The loss of absorbance due to oxidation of NADPH was followed. OHA, N-methyl OHA, and N-benzyl OHA gave similar time courses of inactivation but at concentrations of about 1 μM. Similarly, the methyl ester of OHA and the N-methoxy OHA exhibited time dependent inactivation, but at still higher concentrations (approximately 10^{-5} and 10^{-4} M, respectively). Inhibition of the enzyme was complete at these inhibitor concentrations after incubation for a sufficient duration. If the enzyme was incubated with OHA prior to assay, complete inhibition was observed upon initiating the assay by addition of substrate if Mg^{2+} was present during the pre-incubation. Dilution of the enzyme-OHA complex 100-fold into the assay solution did not reverse the inhibition within a 10-hour period.

This demonstrates that these oxalylhydroxamate derivatives are functionally irreversible inhibitors. By contrast, inhibition of KARI by oxamate (XI, above), acetohydroxamate (X), or pyruvylhydroxamate(XIII), was not time dependent, and gave 50 % inhibition at concentrations of 10^{-3} M for oxamate and acetohydroxamate and 10^{-4} M for pyruvylhydroxamate. This demonstrates that these latter inhibitors are reversible.

Examples 5-16

In Vitro Inhibition of E. coli and Arabidopsis thaliana Ketol-Acid Reductoisomerase

The in vitro inhibition of KARI from bacteria (E. coli) and plants (Arabidopsis thaliana) by N-alkyl oxalylhydroxamates and the corresponding esters is demonstrated in Examples 5-16. E. coli KARI was purified using a modification of the procedure of Arfin et al., J. Biol. Chem. 244:1118 (1969). E. coli and Arabidopsis KARI activity was measured by the radiometric assay for KARI disclosed in the present invention.

This assay measures the time-dependent increase in acid-stable radioactivity due to reduction of [1-^{14}C]-acetolactate (acid-sensitive substrate) to [1-^{14}C]-2,3-dihydroxyisovalerate (acid-stable product) by KARI in the presence of NADPH and magnesium. The reaction was initiated by adding enzyme (4×10^{-5} µmol/min contained in a convenient volume, e. g. 10 to 20 µL) to a 1 mL assay solution (final volume) containing 100 mM HEPES-NaOH, pH 7.4, 10 mM $MgCl_2$, 400 µM NADPH, 125 µM [1-^{14}C]-acetolactate (48 cpm/nmole), and various concentrations of the inhibitor to be tested. After incubating the samples 5 hours at 26°C, the enzymatic reaction was terminated and acetolactate was converted

to acetoin and CO_2 by adding 0.1 mL 50% trifluoracetic acid, with liberation of the radiolabel as $^{14}CO_2$. The solution was evaporated at 100°C under a stream of nitrogen, resuspended in acid, and re-evaporated. The resulting solid was resuspended in 1 mL water, then 10 mL Scintiverse I® (Fischer Scientific) was added, and radioactivity was measured by scintillation counting. The IC_{50} is the concentration of inhibitor that gives 50 % inhibition under these conditions. The results of these experiments are listed below in Table 1.

Although all of the compounds tested were effective inhibitors for both the E. coli and Arabidopsis KARI, the best IC_{50} value for the E. coli KARI under the assay conditions was 9 nM, and the best IC_{50} value for the plant enzyme was 50 nM. Given the complex, time-dependent nature of these inhibitors, as shown in Example 4, fixed-time inhibition values, such as these IC_{50} values, tend to underestimate the potency of the inhibitor.

Table 1

Inhibition of Ketol-Acid Reductoisomerase in Arabidopsis Extracts and with Purified E. coli Ketol-Acid Reductoisomerase

Example	Compound	E. coli IC_{50} (μM)	Arabidopsis IC_{50} (μM)
5	OHA	0.02	0.10
6	N-methyl OHA	0.03	0.10
7	N-methyl OHA methyl ester	0.06	0.35
8	N-ethyl OHA	0.02	0.05
9	N-ethyl OHA methyl ester	0.08	0.25
10	N-isopropyl OHA	0.014	0.25
11	N-isopropyl OHA methyl ester	0.04	0.30
12	N-benzyl OHA	0.009	0.45
13	N-methoxy OHA	0.4	30
14	N-methoxy OHA methyl ester	14	90
15	N-t-butoxy OHA	40	130
16	N-t-butoxy OHA methyl ester	60	400

Examples 17-20

Growth Inhibition of E. coli M152

Growth inhibition of E. coli M152 by N-alkyl oxalylhydroxamates and their methyl esters is

demonstrated in Table 2. Although the M152 strain of E. coli was used due to its simple nutritional requirements (i.e., no amino acids are required for growth), there is no reason that any microorganism that can grow in the absence of branched-chain amino acids could not have been used.

Growth experiments with E. coli M152 were conducted under sterile conditions in culture tubes containing 1.8 mL minimal medium M63 (Difco Laboratories Inc., Detroit, MI) supplemented with 0.01 % thiamine and 0.2 % glucose. Filter-sterilized inhibitor solutions were added to the autoclaved medium to yield final concentrations ranging from 1 µM to 10 mM. Bacteria (0.2 mL of E. coli M152) in stationary phase, which had been cultured overnight in minimal medium containing 0.2 % glucose and 0.01 % thiamine, were added to fresh medium with or without (control) inhibitor. Samples were initially clear but the turbidity of samples lacking inhibitor (controls) increased with time due to the increase in cell mass. The bacteria were grown in an incubator-shaker at 37°C and 250 rpm. Bacterial growth was qualitatively scored as the increase in turbidity relative to the control containing no herbicide. The lowest concentration of inhibitor at which no growth occurred at 8, 19, and 27 hours after the tubes were inoculated with bacteria is listed in Table 2. A concentration-dependent lag in growth was observed at levels of inhibitors greater than 1 µM.

Of the compounds tested, the N-isopropyl OHA derivative was the most potent because effects on the growth lag were observed at micromolar levels.

Table 2

Concentration and Time Dependency of Growth Inhibition of E. coli M152

Example	Compound	Hour post-inoculation at which no growth was observed at the concentration shown		
		8	19	27
17	N-ethyl OHA	1 mM	10 mM	
18	N-isopropyl OHA	1 mM	1 mM	10 mM
19	N-methyl OHA methyl ester	1 mM	10 mM	10 mM
20	N-methoxy OHA	10 mM		

Examples 21-22

Prevention of N-Alkyl Oxalylhydroxamate-Mediated Inhibition of E. coli M152 Growth by Branched-Chain Amino Acids

Prevention of E. coli M152 growth inhibition by N-alkyl OHA with branched-chain amino acids is demonstrated in Examples 21-22.

E. coli cells were grown using conditions listed for Examples 17-20, except some samples containing 1 μM or 1 mM N-isopropyl OHA were supplemented with sterile valine and isoleucine solutions to a final concentration of 1.5 mM. Growth was determined visually in a qualitative fashion, as the increase in turbidity. The results, listed in Table 3, demonstrate valine and isoleucine prevent the inhibition of bacterial growth by 1 μM and 1 mM N-isopropyl OHA.

This illustrates the selective inhibition of bacterial branched-chain amino acid biosynthesis by N-alkyl OHA in intact organisms.

Table 3

Prevention of N-Alkyl Oxalylhydroxamate-Mediated Inhibition of E. coli M152 Growth by Branched-Chain Amino Acids

Example	Compound	conc. cmpd. (mM)	amino acid (1.5 mM)	Growth (turbidity) 3 hr	6 hr	8 hr
	none	-	none	++	+++	++++
21	N-isopropyl OHA	0.001	none	-	-	-
	N-isopropyl OHA	0.001	Ile	-	+	++++
	N-isopropyl OHA	0.001	Ile, Val	++	+++	++++
22	N-isopropyl OHA	1.0	Ile	-	-	-
	N-isopropyl OHA	1.0	Ile, Val	++	+++	++++

Ile = isoleucine; Val = valine

Examples 23-30

Inhibition of Arabidopsis thaliana Growth by Oxalylhydroxamate Derivatives and the Prevention of Inhibition by Branched-Chain Amino Acids

Examples 23, 25-30 in Table 4 illustrate the herbicidal effect of N-alkyl and N-alkylaryl oxalylhydroxamates on Arabidopsis thaliana, and Example 24 demonstrates the prevention of the herbicidal effect by the presence of branched-chain amino acids.

The Arabidopsis assay for herbicide sensitivity is the same procedure described by Haughn et al., Mol. Gen. Genet 204:430 (1986). Seeds were surface sterilized with 25% bleach and 0.02% Triton-X100 then transferred to bacteriological petri plates (90 mm x 23 mm) containing minimal medium and 8 g/L agar to a density of 10 or 19 seeds per plate. Filter-sterilized solutions of OHA compounds and amino acids were added to the desired concentrations after the medium was autoclaved. The seeds were next placed in a dark room

for 3-4 days for etiolation to occur and then transferred to a growth room. The herbicidal effect of the OHA compounds was scored as the % mortality of plants in the presence of OHA compounds relative to control plants in the absence of OHA compounds. Seeds germinated, but the resulting plants died in the presence of OHA compounds. The fraction of plants that died is listed as % mortality in Table 4.

Table 4

Growth Inhibition of Arabidopsis thaliana

Example	Compound	Conc. (ppm)	Presence of Ile and Val (1.5 mM)	% Mortality
23	N-isopropyl OHA	100	-	> 80
24	N-isopropyl OHA	100	+	< 10
25	N-isopropyl OHA	10	-	50
26	N-methyl OHA methyl ester	100	-	> 90
27	N-methyl OHA methyl ester	10	-	< 50
28	N-methoxy OHA	100	-	> 90
29	N-benzyl OHA	100		50
30	N-benzyl OHA	10	-	20

Ile = isoleucine; Val = valine; + = presence of 1.5 mM each of Ile and Val; - = the absence of Ile and Val

Examples 31-37

Specificity of the Inhibition of Plant Ketol-Acid Reductoisomerase in the Branched-Chain Amino Acid Biosynthetic Pathway

Data showing the insensitivity of the other common enzymes, excluding KARI, in the branched-chain amino acid biosynthetic pathway is given in Table 5.

Acetolactate synthase activity was measured by the continuous assay method described by Schloss, et al., Biochemistry 24:4952 (1985). Acetolactate synthase (10 µL) was added to a 1 mL assay solution equilibrated at 25°C containing 100 mM Tricine-NaOH (pH = pK), 50 mM sodium pyruvate, 10 mM $MgCl_2$, 0.1 mM thiamine pyrophosphate, 0.1 mM FAD, and inhibitor. The decrease in pyruvate concentration was monitored at 333 nm for at least 10 minutes.

α,β-Dihydroxyacid dehydratase activity was measured by a modification of the fixed-time assay described by Kiritani and Wagner, Methods Enzymo. 17A:755 (1970). Assay solution (1 mL) containing 50 mM Tris-HCl (pH = 8.0), 10 mM $MgCl_2$, 100 mM α,β-dihydroxyisovalerate, enzyme, and inhibitor was incubated for 30 minutes at 37°C. Trichloroacetic acid (0.25 mL of a 10 % solution) was added to stop the reaction and 0.5 mL saturated 2,4-dinitrophenylhydrazine (in 2 N HCl) was added to form the hydrazone derivative of the α-keto acid produced in the enzymic reaction. The mixture was incubated 10 minutes at room temperature, then 1.75 mL of 2.5 N NaOH was added to solubilize the hydrazone and precipitate unreacted 2,4-dinitrophenylhyrazine. Samples were degassed 10 minutes, centrifuged for 2 minutes, and their absorbance at 550 nm recorded.

The results of these experiments illustrate the insensitivity of the other common enzymes that are involved in branched-chain amino acid biosynthesis to N-alkyl OHA derivatives. These data, together with the data in Examples 21-30, demonstrate the selective inhibition of KARI in intact organisms (i. e., prevention of inhibition by valine and isoleucine), and they demonstrate that KARI is the only enzyme in this pathway that is sensitive to N-alkyl OHA.

Table 5

Specificity of the Inhibition in the Branched-Chain Amino Acid Biosynthetic Pathway

Example	Enzyme	Source	Inhibitor	Conc. (mM)	% Inhib.
31	acetolactate synthase II	E. coli	OHA	0.1	< 5
32	acetolactate synthase II	E. coli	N-isopropyl OHA	1.0	< 5
33	acetolactate synthase II	E. coli	N-isopropyl OHA methyl ester	1.0	< 5
34	dihydroxy-acid dehydratase	Spinach	OHA	1.0	< 5
35	dihydroxy-acid dehydratase	Spinach	N-methyl OHA	1.0	< 5
36	dihydroxy-acid dehydratase	Spinach	N-isopropyl OHA	0.1	< 5
37	dihydroxy-acid dehydratase	Spinach	N-isopropyl OHA	1.0	16

Examples 38-46

Herbicidal Activity of N-Alkyl Oxalylhydroxamate Derivatives

Herbicidal activity of N-alkyl OHA derivatives was also shown by topical application of a herbicide solution to growing plants. Arabidopsis thaliana (a broadleaf plant), Brassica kaber (a broadleaf plant), and Echinochloa crus-galli (a barnyard grass) were grown in pots containing Metro-mix® 350 (W. R. Grace & Co., Cambridge, MA) for at least 1 week before topical application. Several OHA herbicidal solutions were applied as a fine mist to a final concentration of 10, 2, and 0.4 kilograms/hectare of the herbicide. Effects

were recorded after one week and included toxic effects such as plant death, chlorosis, leaf curling, and leaf burn.

Of the herbicides tested, which are listed in Table 6, the N-isopropyl OHA methyl ester was the most potent with plant death observed at 10 kg/hectare for A. thaliana and deleterious effects of the herbicide observed at 0.4 kg/hectare. (No effect was seen in barnyard grass, which was probably due to the higher sensitivity of broadleaf plants.)

Table 6

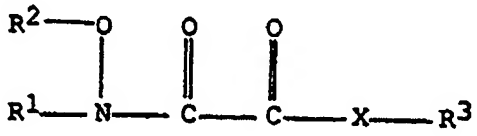
Herbicidal Activity of N-Alkyl Oxalylhydroxamate Derivatives

Example	Plant	inhibitor	[inhibitor] (kg/hectare)	inhibitor effects after 1 week
38	A. thaliana	N-isopropyl OHA methyl ester	10	leaf burn, leaf curling, chlorosis, plant death
39	A. thaliana	N-isopropyl OHA methyl ester	2	leaf curling, chlorosis
40	A. thaliana	N-methyl OHA methyl ester	10	chlorosis
41	B. kaber	N-isopropyl OHA methyl ester	10	leaf burn, leaf curling, chlorosis
42	B. kaber	N-isopropyl OHA methyl ester	2	leaf curling, chlorosis
43	B. kaber	N-isopropyl OHA methyl ester	0.4	leaf curling, chlorosis
44	B. kaber	N-methyl OHA methyl ester	10	chlorosis
45	barnyard grass	N-isopropyl OHA methyl ester	10	no effect
46	barnyard grass	N-methyl OHA methyl ester	10	no effect

CLAIMS

What is claimed is:

1. A process for inhibiting the enzymatic activity of ketol-acid reductoisomerase to effect herbicidal activity, which comprises contacting a plant with an effective amount of a chemical compound of the formula



or an agriculturally acceptable salt thereof, wherein

R^1 is selected from the group consisting of H, alkyl, alkenyl, alkynyl, aryl, alkylaryl, alkenylaryl, and alkynylaryl substituents having from 1 to about 300 atoms, wherein one or more carbon atoms can be optionally substituted with a heteroatom selected from the group consisting of N, S, O, Si, and P, and wherein one or more hydrogen atoms can be optionally substituted with a substituent selected from the group consisting of F, Cl, Br, I, and OH;

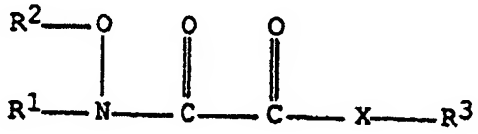
R^2 is selected from the group consisting of the group consisting of H, alkyl, alkenyl, alkynyl, aryl, alkylaryl, alkenylaryl, and alkynylaryl substitutents having from 1 to about 50 atoms, and acyl substituents having from 1 to about 300 atoms, wherein one or more carbon atoms can be optionally substituted with a heteroatom selected from the group consisting of N, S, O, Si, and P, and wherein one or more hydrogen atoms can be optionally substituted with a substituent selected from the group consisting of F, Cl, Br, I, and OH;

R^3 is selected from the group consisting of H, alkyl, alkenyl, alkynyl, aryl, acyl, alkylaryl, alkenylaryl, and alkynylaryl substitutents having from 1 to about 300 atoms, wherein one or more carbon atoms can be optionally substituted with a heteroatom selected from the group consisting of N, S, O, Si, and P, and wherein one or more hydrogen atoms can be optionally substituted with a substituent selected from the group consisting of F, Cl, Br, I, and OH;

and X is selected from the group consisting of O, S, and NY, wherein Y is selected from the group consisting of H, OH, and independently selected R^3;

provided that the combination of X and R^3 is hydrolytically labile.

2. A process for inhibiting the enzymatic activity of ketol-acid reductoisomerase to inhibit bacterial growth, which comprises contacting a microorganism with an effective amount of a chemical compound of the formula



or an agriculturally acceptable salt thereof, wherein

R^1 is selected from the group consisting of H, alkyl, alkenyl, alkynyl, aryl, alkylaryl, alkenylaryl, and alkynylaryl substituents having from 1 to about 300 atoms, wherein one or more carbon atoms can be optionally substituted with a heteroatom selected from the group consisting of N, S, O, Si, and P, and wherein one or more hydrogen atoms can be optionally substituted

with a substituent selected from the group consisting of F, Cl, Br, I, and OH;

R^2 is selected from the group consisting of the group consisting of H, alkyl, alkenyl, alkynyl, aryl, alkylaryl, alkenylaryl, and alkynylaryl substitutents having from 1 to about 50 atoms, and acyl substituents having from 1 to about 300 atoms, wherein one or more carbon atoms can be optionally substituted with a heteroatom selected from the group consisting of N, S, O, Si, and P, and wherein one or more hydrogen atoms can be optionally substituted with a substituent selected from the group consisting of F, Cl, Br, I, and OH;

R^3 is selected from the group consisting of H, alkyl, alkenyl, alkynyl, aryl, acyl, alkylaryl, alkenylaryl, and alkynylaryl substitutents having from 1 to about 300 atoms, wherein one or more carbon atoms can be optionally substituted with a heteroatom selected from the group consisting of N, S, O, Si, and P, and wherein one or more hydrogen atoms can be optionally substituted with a substituent selected from the group consisting of F, Cl, Br, I, and OH;

and X is selected from the group consisting of O, S, and NY, wherein Y is selected from the group consisting of H, OH, and independently selected R^3;

provided that the combination of X and R^3 is hydrolytically labile.

3. A process according to Claim 1 wherein R^1 is joined to either R^2 or R^3 to form a ring.

4. A process according to Claim 1 wherein R^2 is joined to either R^1 or R^3 to form a ring.

5. A process according to Claim 1 wherein R^1 and R^2 and R^3 are joined to form two fused rings.

6. A process according to Claim 1 wherein R^1 has from 1 to about 150 atoms, R^2 has from 1 to about 50 atoms, and R^3 has from 1 to about 150 atoms.

7. A process according to Claim 6 wherein R^1 has from 1 to about 100 atoms, R^2 has from 1 to about 30 atoms, and R^3 has from 1 to about 100 atoms.

8. A process according to Claim 7 wherein R^1 has from 1 to about 50 atoms, R^2 has from 1 to about 20 atoms, and R^3 has from 1 to about 50 atoms.

9. A process according to Claim 8 wherein X is O.

10. A process according to Claim 9 wherein R^1 is selected from the group consisting of hydrogen, methyl, ethyl, isopropyl, t-butyl, and benzyl.

11. A process according to Claim 10 wherein R^1 is selected from the group consising of hydrogen, methyl, ethyl, isopropyl, and benzyl.

12. A process according to Claim 9 wherein R^2 is selected from the group consisting of hydrogen, methyl, and t-butyl.

13. A process according to Claim 12 wherein R^2 is hydrogen.

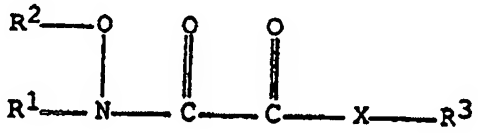
14. A process according to Claim 9 wherein R^3 is selected from the group consisting of hydrogen and methyl.

15. A process according to Claim 14 wherein R^3 is hydrogen.

16. A process according to Claim 9 wherein the compound is selected from the group consisisting of N-isopropyl oxalylhydroxamate, N-ethyl oxalylhydroxamate, and N-methyl oxalylhydroxamate.

17. A process according to Claim 16 wherein the compound is N-isopropyl oxalylhydroxamate.

18. A chemical compound of the formula



wherein

R^1 is selected from the group consisting of H, methyl, ethyl, propyl, isopropyl, butyl, sec-butyl, isobutyl, t-butyl, phenyl, 2-pentyl, 3-pentyl, 2-hexyl, 3-hexyl, C1-C6 perfluoroalkyls, and benzyl wherein one or more o-, m-, or p- positions can be optionally substituted with a substituent selected from the group consisting of F, Cl, Br, I, methoxy, methyl, and trifluoromethyl;

R^2 is selected from the group consisting of H, methyl, ethyl, propyl, isopropyl, butyl, sec-butyl, isobutyl, t-butyl, carbamoyl, N,N-dimethylcarbamoyl, N,N-dimethylthiocarbamoyl, N,N-diphenylcarbamoyl, N,N-diphenylthiocarbamoyl, acetyl, thioacetyl, propanoyl, thiopropanoyl, trimethylsilyl, t-butyldimethylsilyl, and benzoyl wherein one or more o-, m-, or p- positions can be optionally substituted

with a substituent selected from the group consisting of F, Cl, Br, I, methoxy, methyl, and trifluoromethyl;

R^3 is selected from the group consisting of H, methyl, propyl, isopropyl, butyl, sec-butyl, isobutyl, t-butyl, 2-pentyl, 3-pentyl, 2-hexyl, 3-hexyl, and C1-C6 perfluoroalkyls;

and X is selected from the group consisting of O and S;

provided that when R^1 is H, R^2 cannot be H; and further provided that when R^2 is H, R^1 and R^3 cannot both be methyl.

19. A compound according to Claim 18 wherein R^1 is ethyl and R^2 is carbamoyl and C-2 of ethyl and N of carbamoyl are joined to form a ring.

20. A compound according to Claim 18 wherein R^1 is selected from the group consisting of isopropyl and ethyl.

21. A compound according to Claim 20 wherein R^1 is ethyl.

22. A compound according to Claim 18 wherein R^2 is H.

23. A process for assaying ketol-acid reductoisomerase, which comprises

mixing an aliquot selected from the group consisting of a plant extract and a microbial extract with radioactive [1-^{14}C]-acetolactate, NADPH, and Mg^{2+}, in a buffered solution having a pH above about 6 and below about 9;

adding an acid to the assay mixture, after an appropriate incubation time;

removing volatile components from the acidified mixture; and measuring the remaining radioactivity.